

EXHIBIT B

Neointimal Hyperplasia After Arterial Injury Is Increased in a Rat Model of Non-Insulin-Dependent Diabetes Mellitus

Si-Hoon Park, MD; Steven P. Marso, MD; Zhongmin Zhou, MD; Farhard Foroudi, BS;
Eric J. Topol, MD; A. Michael Lincoff, MD

Background—The key biological determinants that promote restenosis in the setting of diabetes have not been elucidated. There is no accepted animal model to study restenosis in diabetes.

Methods and Results—We evaluated 2 models of diabetes mellitus: (1) streptozotocin (STZ)-treated Sprague-Dawley rats (type I diabetes) versus regular Sprague-Dawley rats and (2) obese Zucker rats (type II diabetes) versus lean Zucker rats. Neointimal hyperplasia was assessed after carotid balloon injury at 21 days by computerized morphometry. There was no difference in neointimal area in the STZ-treated rats compared with controls, irrespective of insulin administration or dose of STZ. Neointimal area was increased >2-fold in obese Zucker rats compared with lean Zucker rats (0.21 ± 0.06 versus $0.08 \pm 0.03 \text{ mm}^2$, $P < 0.01$). The neointimal area was markedly increased in the obese Zucker rats 7 days after injury (0.058 ± 0.024 versus $0.033 \pm 0.009 \text{ mm}^2$, $P < 0.05$) and persisted through 21 days. In both obese and lean Zucker rats, cell proliferation peaked in the media at 3 days (118.66 ± 84.28 versus 27.50 ± 12.75 bromodeoxyuridine-labeled cells per cross section). In the intima, cell proliferation markedly increased beginning at day 3 and persisted through day 14 in the obese and lean Zucker rats (202.27 ± 98.86 versus 35.71 ± 20.54 bromodeoxyuridine-labeled cells at 7 days).

Conclusions—The type II diabetic rat model, typifying insulin resistance, is associated with a propensity for neointima. The obese Zucker rat model may be an ideal diabetic model to further characterize the diabetic vascular response to injury. (*Circulation*. 2001;104:815-819.)

Key Words: diabetes mellitus ■ restenosis ■ insulin ■ angioplasty

Diabetic patients have an increased incidence of acute complications, late myocardial infarction, restenosis, and mortality after percutaneous coronary intervention.¹⁻⁵ Intravascular ultrasound studies demonstrate that neointimal proliferation, after both balloon angioplasty and stenting, results in greater restenosis rates in diabetic patients. The biological determinants of restenosis among patients with diabetes mellitus, however, are unknown. Therefore, we developed type I and II diabetic animal models of arterial injury to investigate the pathobiology of restenosis in diabetic patients. We hypothesized that both diabetic models would have enhanced neointimal proliferation compared with control animals. We also examined potential biological contributors to coronary restenosis in diabetes, such as insulin resistance, exogenous insulin administration, and glucose, cholesterol, and triglyceride levels. We characterized the mechanisms of the pathogenesis of restenosis in diabetes with regard to smooth muscle cell proliferation.

Methods

Type I (Insulin-Dependent) Diabetic Animals

To make a type I diabetic rat model, we injected streptozotocin (STZ) into Sprague-Dawley rats, followed by arterial injury 2 weeks

later. Male Sprague-Dawley rats 11 to 14 weeks old were selected at random for intravenous injection of STZ in 50 mmol/L citrate buffer (pH 4.5) or citrate buffer alone (control group). Animals were weighed immediately before STZ injection. We used 2 different doses of STZ, a relatively low dose (40 mg/kg) and a high dose (60 mg/kg), to simulate 2 diabetic rat groups with various degrees of insulin deficiency.^{6,7} These 2 type I diabetic rat groups were each compared with separate control groups in separate experiments. STZ-injected rats were further divided into 2 groups according to whether or not they received insulin treatment. Rats in the insulin-treated diabetic cohorts were treated subcutaneously with 2 U NPH insulin for the low-dose STZ group and 4 U NPH insulin for the high-dose group, beginning 1 week after injection of STZ. All animals were allowed free access to food and water. Animals were reweighed weekly. A blood glucose level of ≥ 250 mg/dL, documented 1 week after injection of STZ, was required for inclusion of the diabetic animal in the study. Animals were euthanized 3 weeks after arterial injury for assessment of neointimal hyperplasia.

Type II (Insulin-Resistant) Diabetic Animals

The obese Zucker rat, characterized by excessive body weight, insulin resistance, hyperinsulinemia, and mild hyperglycemia, is a well-established model of type II diabetes.⁸ We included obese Zucker rats 8 to 9 weeks old for a type II diabetic model and lean Zucker rats 11 to 14 weeks old as controls. Experiments were

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From the Department of Cardiology, The Cleveland Clinic Foundation, Cleveland, Ohio (S.P., Z.Z., E.J.T., A.M.L.), and Mid America Heart Institute, Saint Luke's Hospital, Kansas City, Mo (S.P.M.).

The first 2 authors contributed equally to this study.

Correspondence to A. Michael Lincoff, MD, Department of Cardiology, F25, The Cleveland Clinic Foundation, 9500 Euclid Ave, Cleveland, OH 44195-5066.

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performed in 2 separate sessions. We first evaluated neointimal hyperplasia 3 weeks after arterial injury and then, in separate experiments, we assessed cell proliferation at 1, 3, 7, 14, and 21 days after injury.

Surgical Procedure and Tissue Preparation

Arterial injury was performed by balloon deendothelialization. After induction of anesthesia with an intraperitoneal injection of xylazine (4.6 mg/kg) and ketamine (70 mg/kg), a midline cervical incision was made to expose the left external carotid artery. With a 2F Fogarty balloon catheter (Baxter Healthcare Corp), carotid artery injury was performed according to a previously described technique.⁹ After anesthesia, animals were euthanized through a midline abdominal incision exposing the distal abdominal aorta. With an 18-gauge intravenous catheter introduced at the aortic bifurcation, the aorta was flushed with 50 mL of Ringer's lactate solution at 120 mm Hg, followed by *in vivo* fixation with 200 mL of 5% Histochoice (Amresco) infused over 5 minutes at 120 mm Hg. Histochoice is an acid aldehyde with 10% alcohol solution. Once the perfusion-fixation was started, the animals were killed with an overdose of thiopental sodium through the tail vein. After 5 minutes of perfusion-fixation, the entire left carotid arteries were harvested, including the aortic arch, innominate artery, and carotid bifurcation. The specimens were stored in 5% Histochoice until sectioning. The injured common carotid arteries were cut every 3 mm from the aortic arch to the bifurcation into the external and internal carotid arteries. Five segments were embedded in paraffin for sectioning, and duplicate slides were stained with hematoxylin-eosin and elastic van Gieson stain. Three different segments, with the maximal neointimal proliferation of the left common carotid artery, were selected for histological, morphometric, and immunohistochemical studies.

Histomorphometric Study

Morphometric analysis of the arterial segments was carried out by an observer blinded to the study group, and quantitative measurements were performed on the segment that exhibited the greatest area of neointima. With a computerized digital microscopic planimetry algorithm (NIH Image 1.56), cross-sectional areas of the lumen, intima, media, and vessel circumscribed by the external elastic lamina were measured. Intimal cell counting was performed with a previously validated method.¹⁰ Analyses were done on cross sections stained with hematoxylin-eosin under $\times 40$ microscopic magnification. Random areas (encompassing 20% to 40% of the total intimal cross-sectional area) within the intima were selected, and cell nuclei were enhanced and counted after dynamic color thresholding. The average cell nuclear count within these known areas was used to calculate the cell density (cells/mm²), which, when multiplied by the previously measured total intimal area (from elastic van Gieson-stained sections), was used to calculate the total intimal cell count.

BrdU Injection and Immunohistochemistry

To detect proliferative cells at each time point after vascular injury, we injected bromodeoxyuridine (BrdU) (Sigma Chemical Co) and performed immunohistochemistry¹¹ at each of the 5 time points after balloon injury. The BrdU antibody dilution was 1:100. BrdU was administered at 18 hours (30 mg/kg IP and 100 mg/kg SC) and 12 hours (30 mg/kg IP) before euthanasia. Immunohistochemistry was performed with a monoclonal antibody (Dako Co) to BrdU. BrdU-labeled cells were counted in media and intima at $\times 200$ magnification. In the intima, the BrdU-labeling index (the fraction of labeled nuclei times 100) was calculated to assess cell replication rate. The temporal and spatial presence of BrdU-labeled cells was observed in the media and intima to assess cell migration after carotid balloon injury.

Blood Chemistry Assay

Blood glucose levels were checked twice weekly for 2 weeks after injection of STZ and weekly thereafter. Blood glucose was measured with a standardized, portable glucometer in blood obtained from the tail vein. Serum was obtained from nonfasted animals at death and

frozen at -20°C until assay. Total cholesterol was measured by the cholesterol oxidase enzyme assay, triglycerides by the glycerol triphosphate oxidase enzyme assay, and insulin levels by radioimmunoassay with an antibody made specifically against rat insulin (Linco). The rat insulin antibody has 100% cross-reactivity with porcine insulin. Ketone bodies were checked by the nitroprusside reaction.

Statistical Analysis

All data were expressed as mean \pm SD. All statistical analyses were performed with Stat-View Version 4.5 (Abacus Concepts, Inc). The unpaired Student's *t* test and Fisher's exact test were used to compare parametric data and nonparametric data, respectively, between 2 groups. When comparisons of >2 groups were required, statistical significance was determined by ANOVA. A value of $P < 0.05$ was considered significant.

Results

Sixty-two obese Zucker rats, 60 lean Zucker rats, and 140 Sprague-Dawley rats were started on the experimental protocol. A total of 55 obese Zucker rats and 55 lean Zucker rats survived until the time of euthanasia. Twelve Sprague-Dawley rats were excluded for inadequate glucose levels after STZ injection, 28 died during surgery, and an additional 15 died in the follow-up period. A total of 85 Sprague-Dawley rats thus survived until euthanasia. The type I diabetic rats without exogenous insulin treatment exhibited marked weight loss, which was related to the dose of STZ (Table 1). The glucose, triglyceride, cholesterol, and insulin levels are depicted in Table 1.

Morphometry at 21 Days After Carotid Balloon Injury in Diabetic Rats

In the type I diabetic rats, irrespective of insulin treatment and dose of STZ, there were no significant differences in neointimal hyperplasia 21 days after carotid balloon injury compared with controls. There were also no differences in lumen, external elastic lamina, or medial areas (Table 2). In contrast, the neointimal area was increased >2 -fold in the type II diabetic obese Zucker rats compared with control lean Zucker rats (0.21 ± 0.06 versus 0.08 ± 0.03 mm², $P < 0.01$). There were no differences in luminal or medial areas between obese and lean Zucker rats, but the external elastic lamina area was larger in obese Zucker rats (Table 2).

Time Course of Neointimal Formation in Type II Diabetic Rats

Compared with lean Zucker rats, neointimal area was increased in type II diabetic obese Zucker rats beginning 7 days after injury (0.058 ± 0.024 versus 0.033 ± 0.009 mm², $P < 0.05$) (Figure 1). Differences in neointimal area became progressively greater in obese versus lean Zucker rats and continued to diverge through 21 days (0.20 ± 0.03 versus 0.10 ± 0.03 mm², $P < 0.01$).

Cell Proliferation

The number of proliferating BrdU-labeled cells in the media was highest in obese Zucker rats relative to lean Zucker rats at 3 days (118.66 ± 84.28 versus 27.50 ± 12.75 cells per cross section, $P < 0.05$) and remained significantly higher in obese Zucker rats at 7 days (29.36 ± 26.44 versus 1.14 ± 1.21 cells per cross section, $P < 0.05$) (Figure 2). In the intima, the

TABLE 1. Animal Characteristics and Laboratory Findings

Zucker Rat	STZ-Injected Rat							
	High Dose (60 mg/kg)				Low Dose (40 mg/kg)			
	OZ (n=12)	LZ (n=14)	STZ+I (n=15)	STZ (n=15)	Control (n=16)	STZ+I (n=13)	STZ (n=13)	Control (n=13)
Weight, g								
Initial	373±9	325±25	347±26	333±14	322±3	348±28	332±14	318±6
At death	452±13	363±14	387±31	267±18	382±10	387±32	297±14	379±11
Glucose, mg%	229±21*	173±36	251±41*	428±66*	174±26	221±18*	303±18*	162±19
Triglycerides, mg%	549±241*	71±16	60±23	852±624*	70±15	60±24	134±18*	70±14
Cholesterol, mg%	84±17	63±10	85±21	161±45*	84±7	82±21	103±9*	83±6
Insulin, ng/mL	3.74±3.16*	1.06±0.38	5.99±3.38*	0.58±0.18	1.05±0.42	4.32±1.62*	0.87±0.30*	1.39±0.45
Ketone (+)	0	0	0	13*	0	0	2	0

OZ indicates obese Zucker rat; LZ, lean Zucker rat; STZ+I, streptozotocin-injected rats with insulin treatment; and STZ, streptozotocin-injected rats without insulin treatment.

*P<0.01 vs control group in each diabetic model.

number of BrdU-labeled cells and the BrdU-labeling index was highest in obese Zucker rats relative to control lean Zucker rats at 7 days (202.27 ± 98.86 versus 35.71 ± 20.54 cells per cross section, $P<0.05$; $77.8\pm6.89\%$ versus $40.63\pm6.75\%$ index, $P<0.01$) and significantly higher in obese Zucker rats at 14 days (111.80 ± 30.44 versus 54.45 ± 20.22 cells per cross section, $P<0.01$; $47.81\pm8.80\%$ versus $38.80\pm7.82\%$ index, $P<0.05$) (Figure 3). By 21 days after injury, cell proliferation in the intima of obese Zucker rats had diminished to the level of lean Zucker rats. In both obese and lean Zucker rats, cell proliferation peaked in the media at 3 days (118.66 ± 84.28 and 27.50 ± 12.75 BrdU-labeled cells per cross section). In the intima, cell proliferation was markedly increased at 3 days and persisted through days 7 and 14 in both the obese and lean Zucker rats (202.27 ± 98.86 and 35.71 ± 20.54 BrdU-labeled cells per cross section at 7 days).

Discussion

Restenosis remains a major limitation of percutaneous coronary revascularization for patients with diabetes, and the factors contributing to this risk remain poorly defined. In this study, we assessed both type I and type II rat models for diabetes. There was not an increased propensity to form neointima in the type I diabetic rat model compared with

controls. The Zucker rat model, which is a prototype for type II diabetes, however, exhibited a marked tendency to form neointima after balloon injury compared with the lean controls. In both models, the rats had increased levels of serum glucose, cholesterol, and triglycerides, which were attenuated with exogenous insulin administration in the type I model compared with control rats. There were also increased circulating insulin levels in the type II and insulin-treated type I models compared with respective controls.

Diabetes and Restenosis

Patients with a reported history of diabetes mellitus account for 15% to 25% of those undergoing percutaneous coronary intervention procedures. Well over 90% of these patients have type II diabetes.

Unfortunately, there are currently no adequately characterized animal models to investigate the vascular response to injury in diabetes.

Generally, restenosis after balloon injury is a function of early elastic recoil, adverse remodeling, and the formation of neointima. The advent of stenting has favorably affected the rate of restenosis among diabetic and nondiabetic patients, primarily by reducing elastic recoil and adverse remodeling. Even in the present stent era, diabetes is associated with an increased risk of restenosis. This is a direct result of the

TABLE 2. Morphometry 21 Days After Carotid Balloon Injury in Each Diabetic Rat Model

Zucker Rat	STZ-Injected Rat							
	High Dose (60 mg/kg)				Low Dose (40 mg/kg)			
	OZ (n=12)	LZ (n=14)	STZ+I (n=15)	STZ (n=15)	Control (n=16)	STZ+I (n=13)	STZ (n=13)	Control (n=13)
Intima, mm ²								
Intima	0.21±0.06*	0.08±0.03	0.09±0.04	0.09±0.04	0.11±0.03	0.10±0.04	0.08±0.04	0.10±0.05
Media, mm ²	0.13±0.03	0.12±0.02	0.12±0.02	0.12±0.03	0.13±0.02	0.13±0.02	0.12±0.02	0.13±0.02
Lumen, mm ²	0.23±0.05	0.28±0.09	0.34±0.11	0.33±0.12	0.33±0.11	0.34±0.10	0.34±0.10	0.32±0.11
External elastic lamina, mm ²	0.58±0.01†	0.48±0.01	0.52±0.09	0.55±0.16	0.57±0.13	0.56±0.10	0.54±0.13	0.56±0.14

Abbreviations as in Table 1.

*P<0.01, †P<0.05 vs control group in each diabetic model.

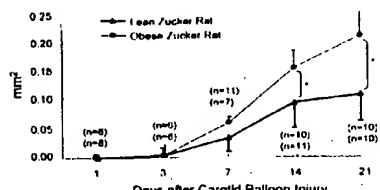


Figure 1. Changes of neointimal area after carotid balloon injury. Neointimal area progressively increased in type II diabetic obese Zucker rats vs lean Zucker rats beginning 7 days after injury. $P<0.01$.

formation of neointima; thus, it has become imperative to develop, characterize, and ultimately modulate a diabetic model that demonstrates a propensity to form neointima. In the diabetic state, several mechanisms, such as enhanced coagulability, platelet hypersensitivity, dyslipidemia, or dysregulation of chemotactic factor expression, might combine to produce enhanced intimal hyperplasia.¹² All these putative mechanisms may be influenced by hyperglycemia, hyperinsulinemia, or insulin resistance.

Previous Animal Studies

There have been conflicting results in previous diabetic animal studies regarding the association of increased neointimal proliferation after arterial injury and the different types of diabetes. Two previous studies in type I diabetic models (Alloxan-induced diabetic rabbit¹³ and BB Wistar diabetic rat¹⁴) demonstrated increased intimal thickening after balloon injury compared with nondiabetic animals. In contradistinction, increased aortic intimal thickening was observed in the obese Zucker type II diabetic rats compared with the low-dose STZ-treated, insulin-treated Wistar diabetic rats.¹⁵ Our data also found an increase in neointimal proliferation after balloon injury in the obese Zucker rats. To characterize this model more completely, we included both insulin-treated rat and insulin-untreated rat groups in our study. There was no association, however, between neointimal hyperplasia and exogenous insulin administration, insulin deficiency, glucose, lipid, or triglyceride levels. Our study suggests an association of intimal hyperplasia and insulin resistance. In short, the underpinnings of proliferation after injury remain undetermined; however, further analysis with an insulin-resistant state seems warranted.

Diabetes results in increased circulating serum glucose levels. Enhanced glucose control has been shown to reduce microvascular complications in both type I¹⁶ and type II patients.¹⁷ Improvement in macrovascular complications, however, was not demonstrated in these trials. Hyperglycemia

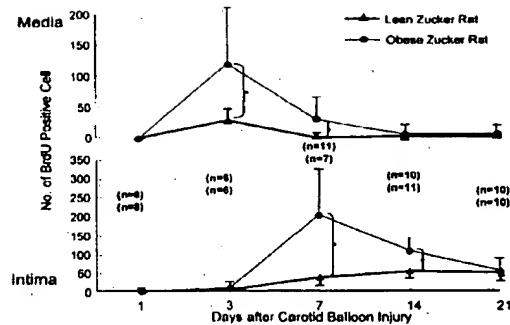


Figure 3. Number of BrdU-positive cells located in intima and media after balloon injury in Zucker obese and lean rats. $P<0.05$.

might potentiate the response to arterial injury and may affect the initial step of restenosis.²¹ There is a significant correlation between thromboxane A₂ production, fasting plasma glucose, or hemoglobin A_{1C} levels and the expression of several growth factors.¹⁹ Hyperglycemia induces an increase in selected matrix gene transcriptions that persists for weeks after restoration of normoglycemia *in vivo*.²⁰ Furthermore, advanced glycosylation end products can mediate inflammatory cell recruitment and activation, stimulation of smooth muscle cell proliferation, and abnormal matrix production, all of which may promote restenosis. The degree of early nonenzymatic glycosylation is determined mainly by the glucose concentration.²¹ Formation of advanced glycosylation end products results from prolonged hyperglycemia and enhanced oxidative state. Inhibition of advanced glycosylation end products has been shown to decrease de novo atherosclerotic formation in the STZ-induced apolipoprotein E-null mouse model.²² Although hyperglycemia is potentially related to many steps in the process of restenosis, at present, no consistent data implicate hyperglycemia and restenosis.

Insulin has several biological actions, which may be related to the process of restenosis. Insulin exerts adverse effects on the balance between thrombosis and fibrinolysis by modulating the plasminogen activator and inhibitor systems.²³ Insulin can potentiate proliferation and migration of smooth muscle cells, most likely through the action of insulin-like growth factor²⁴ or other stimulatory factors.²⁵ Insulin also aggravates diabetic dyslipidemia²⁶ and theoretically promotes long-term recoil of the overstretched arteries.¹⁵ Although insulin and insulin-like growth factors are mitogenic, controversy remains as to the importance of insulin in promoting smooth

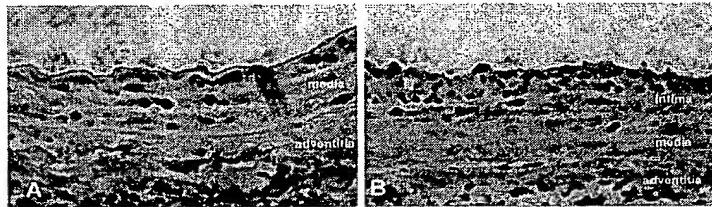


Figure 2. Photomicrograph of proliferative cells in media and intima after carotid balloon injury. Number of BrdU-labeled cells was significantly higher in the obese Zucker rat (B) than the lean Zucker rat (A) after balloon injury.

muscle cell proliferation and whether insulin alone or insulin resistance syndrome is key in modulating restenosis.

Insulin resistance is probably only a modest predictor of macrovascular disease,²⁷ and whether it is associated with increased restenosis is uncertain. Clinically, insulin resistance is characterized by the presence of diabetes, hypertension, obesity, and dyslipidemia. There are also suggestive data in humans that insulin-resistance syndrome is associated with restenosis among nondiabetic patients.^{28,29} Among nondiabetic insulin-resistant patients, Nishimoto et al²⁸ determined that insulin resistance was associated with an increased risk for restenosis after angioplasty. Furthermore, in EPISTENT,³⁰ patients with insulin resistance syndrome (defined as diabetes mellitus, hypertension, and obesity) had a higher rate of 6-month target vessel revascularization than the non-insulin-resistant cohort (16.7% versus 7.5%, $P < 0.001$). Likewise, data for this project would suggest that it is the insulin-resistant state rather than diabetes that results in the formation of neointima.

Study Limitations

This study has several limitations. The relevance of restenotic animal models to human restenosis is unknown, and no single model has yet been shown to reliably predict restenosis in humans. In fact, it is unlikely that any one model will be entirely explanatory of the human response to injury. Animal studies, however, are likely to provide important insights into the pathophysiology of vascular injury.

Conclusions

The obese Zucker rat carotid artery injury model appears to be an appropriate animal model for the study of the mechanism and treatment of increased restenosis associated with type II diabetes. The data from this study would suggest that an insulin-resistant model is associated with a propensity for neointimal proliferation. This was not seen in an insulin-deficient model with or without exogenous insulin administration.

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